IJBST

ISSN: 0974 – 3987
IJBST (2014), 7(4):25-30

The Antimicrobial effect of Natural Bioactive compound isolated from soil fungi against *Klebsiella pneumoniae* and MRSA

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ABSTRACT:
Fungal isolates are receiving increasing attention by natural product chemists due to their diverse and structurally unprecedented compounds making them interesting candidates for drug discovery. Two out of 10 tested bioactive extracts namely MRP001 and MRP014 exhibited the highest antibacterial activity against *Klebsiella pneumoniae* and MRSA. However the bioactive extracts with antibacterial activity showed a little variation in their activity against the tested bacteria. The methanolic extracts of MRP001 exhibited a high potency in terms of MIC and MBC values against the growth of *Klebsiella pneumoniae* and MRSA. Time-kill curve showed a fast and sharp antibacterial activity against *Klebsiella pneumoniae* and MRSA. *Myrothecium* extracts targeted microbial cell membranes and cell walls, and led to structural disorganization of the cell. Methanol extract MRP001 proved to be good candidates for further development as antibacterial agents for infections caused by *Klebsiella pneumoniae* and MRSA and may also oppose the resistance of the conventional used antibacterial agents. Chemical analysis of bioactive extract explored that presence of glycosides, phenol, flavanoids and steroids. Electron microscopy examination of isolated bioactive compound treated cells showed a great variation in the cell structure.

Keywords: Bioactive compound, *Myrothecium sp.*, *Klebsiella pneumonia*, MRSA.

INTRODUCTION

In the last several years, the frequency and spectrum of antimicrobial resistant infections have increased in both the hospital and the community due to the continued use of systemic and topical antimicrobial agents which, in turn, drove the continued search for new agents [1]. In addition, the side effects of overuse and misuse of antibiotics can harm vital organs [2]. Most important multidrug-resistant bacteria on the global scale include gram positive (methicillin-resistant *Staphylococcus aureus*, vancomycin resistant *enterococci*) and gram-negative bacteria (members of *enterobacteriaceae* producing plasmid-mediated extended spectrum beta-lactamase and others as *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* [3]. Nowadays; pharmaceutical companies are developing new antibiotics to replace those that are no longer effective [4]. In recent years we have turned our attention to mining our library of fungi for new bioactive compounds possessing selectivity to antibacterial activity against pathogens [5]. The library is now greater than 1000 strains and provided the platform for the research reported below. We began with the straight forward precept that certain of these strains would produce, under saltwater culture condition, active extracts, and this could be followed up with the bioassay guided isolation to yield significant compounds. Thus, an extensive effort was begun to validate our ideas and obtain chemo types that would be candidates for further pharmacological follow up [6]. Moreover, the whole population of microorganisms is being thought to possess a strikingly bigger biodiversity than those of animal and plant kingdoms. This is why soil microbes are currently receiving much more attention highlighting that some natural microbial products have become challenges both for structural elucidation and synthesis, and for their different bioactivities [7, 8].

In our continuous search for chemically unique and/or biologically potent natural products from soil fungi [9], we observed that the ethyl acetate extract of a fungal strain under the number MRP001 isolated from *Myrothecium species* was not only strongly inhibitory to the growth of the human pathogens fungi (*Candida albicans*, *Aspergillus niger)* and bacteria (*Escherichia coli*, *Salmonella Typhi*, *Klebsiella pneumoniae* and *Bacillus cereus*), but also allergic to human skin.

In this study, we demonstrated that isolated bioactive compound MRP001 inhibited the growth of *Klebsiella pneumoniae* and MRSA. From this result we anticipate the discovery of a new compound that confers resistance to the *Klebsiella pneumoniae* and MRSA.

MATERIALS AND METHODS

Microorganisms

The screening of microorganisms was done against various pathogens and antimicrobial activity found in isolated fungal strain was further confirmed to be *Myrothecium* spp MRP001 in our previous study [10]. The strain *Myrothecium* spp MRP001 which was used for this study was isolated from soil of PDKV region of Akola District, India. The stock culture of strain was maintained on a potato dextrose agar (PDA) slants. Slants were inoculated, incubated at 28 °C for 7 days and then stored at 4 °C.

Two different bacterial isolates were used throughout the present work; one gram negative *Klebsiella*

pneumoniae and the other gram-positive methicillin-resistant *Staphylococcus aureus* were isolated from patients with urinary tract infection and identified phenotypically as described in Beregy's Manual of determinative bacteriology [11].

Chemicals

A soil sample was collected from local area at Akola, Maharashtra, India. All the prepared reagents used in the present investigation were provided by SDFCL, Mumbai. The culture media and the antibiotics used were supplied by Hi-Media Chemicals, Mumbai, India. Other used chemicals of laboratory and analytical grade were obtained from recognized chemical suppliers. (Merck, Mumbai).

Inoculum preparation and standardization

Inoculums were prepared directly by suspending colonies grown overnight on nutrient agar plate directly in sterile saline solution (0.85%). Suspensions were adjusted by using the Macfarland which corresponds approximately to 1.5 x 10^8 CFU/ml [12]. In some experiments bacterial suspension were diluted, or supplemented with more organisms, as needed to correspond to final inoclum concentrations 1.5 x 10^8 CFU/ml [13].

Solvent Extraction Method

The cultural broth was extracted using the method of Accelerated Solvent Extraction (ASE) method. The extraction was conducted consecutively with acetone, dichloromethane (DCM), methanol (MeOH) and deionized water (DWR) [14]. The extract was filtered and the filtrate was concentrated by Rotavapor (R-114) at 60 °C to get an oily residue. The extract was stored in labelled sterile screw capped bottle at 4 °C till further analysis. The extract showing antimicrobial activity against pathogens using In-vitro plate assay was loaded to silica gel column chromatography [15].

Maintenance of the bioactive extracts

All bioactive extracts were tested for their sterility and stored at 25 °C in sterile brown glass containers and placed in dark places to prevent photo-isomerization. Screening of antibacterial activity of bioactive extracts and the effect of some bioactive extracts was tested against the growth of Staphylococcus *aureus* and *Klebsiella pneumoniae* using the well diffusion method. An inoculum of bacterial suspension (1.5 x 10^8 CFU/ml) equivalent to 0.5 McFarland was prepared, and 25 µl were swabbed over the surface of Müller-Hinton agar plate. A 6mm well was cut in the centre of each plate using a sterile cork borer. Twenty five µl of each bioactive extract were pippetted into each well. Plates were placed at 4 °C for 1 h for compound diffusion and then incubated for 24 h at 28 °C. Results of the qualitative screening were recorded as average diameter of the inhibition zone surrounding the wells containing the test solution [16]. All the experiments were done in triplicate.

Determination of minimal inhibitory concentration (MIC) of some selected extracts

A series of dilutions of each tested extract was prepared in a 96-well microdilution tray with an initial extract concentration of 100 % and a final concentration of 10%. The cultures of the bacteria under test were diluted in Müller-Hinton broth at a density adjusted to a 0.5 McFarland turbidity. The final inoculum was 1.5 x 10^8 CFU/ml of bacterial cultures. After the addition of inocula of bacterial broth, trays were covered, incubated at 37 °C for 24 h. MICs were determined visually, according to NCCLS guidelines [17]. The experiment was repeated three times. The MIC is the lowest concentration of extract that inhibited the growth of the test strain in the wells by visual reading and by the growth inhibition on macro-plates as described by Ellof [18].

Determination of minimal bactericidal concentration (MBC) of some selected extracts

The minimum bactericidal concentrations (MBCs) of selected extracts were determined by inoculating the MIC dilution onto Müller-Hinton agar plates and incubated at 37 °C for 18 h [19]. MBCs were determined as the lowest concentration resulting in no growth on subculture.

Determination of bacterial time Kill Curve

A time-Kill curve was assessed to investigate the best time for extracts that kills the bacterial vegetative cells. Therefore, the selected extracts that showed a bactericidal effect against the most promising bacterium under test were used, and survivor (time-kill) curve was plotted. A 16 h culture was harvested by centrifugation. The suspension was adjusted using the McFarland standard and was then further diluted in saline 0.85% to achieve approximately 1.5 x 10^8 CFU/ml. The selected bioactive extracts were added to aliquots of 1 ml Müller-Hinton broth in tubes in water bath at 37 °C in amounts that would achieve bactericidal concentrations for the selected bacteria followed by the addition of 1 ml of the inoculums. Further samples were taken from each tube in order to monitor bacterial growth by measuring the absorbance (optical density) at 600 nm wavelengths at time intervals (0, 2, 4, 6, 8, 12 and 24 h) and incubated at 37 °C (Yin et al., 2002). All the experiments were carried in triplicate.

Screening for testing the antimicrobial combinations of the promising extracts with antibiotics against Klebsiella pneumoniae and MRSA

Seven antibiotic discs were selected. One was used for both gram-negative and gram-positive bacteria; ciprofloxacin (CIP). Two antibiotic discs for gram negative bacteria were used: gentamycin (GM) and

ciproflaxin(CIP). The discs used for gram-positive bacteria were cefoxitin (FOX), oxacillin (OX), ceftriaxone (CRO) and vancomycin (VA) [20]. A McFarland 0.5 standard bacterial suspension was swabbed on the top of the solidified Müller-Hinton agar plates and allowed to dry for 10 min. The discs combined with bioactive extract were placed on the inoculated agar by pressing slightly. The plates were placed at 4 °C for 1 h for compound diffusion and then incubated for 24 h at 37 °C. Zone of inhibition were recorded in millimetres.

CHEMICAL ANALYSIS [21]

Test for Flavonoids (Ammonia test)
The isolated bioactive extract (1ml) was taken in the test tube and ammonia solution was added (1:5) followed by the addition of conc. sulphuric acid. Appearance of yellow color and its disappearance on standing indicates the positive test for flavonoids.

Test for Phenols (Ferric chloride test)
The extract (0.5ml) was added with few drops of neutral ferric chloride (0.5%) solution. Formation of dark green color indicates the presence of the phenolic compounds.

Test for Steroids: (Libermann-Burchard's test)
Acetic anhydride (2ml) was added to 0.5 ml of the bioactive extract and then followed by the addition of 2ml conc. sulphuric acid slowly along the sides of the test tube. Change of colour from violet to blue or green indicates the presence of steroids.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

On the basis of MIC, MBC values and Time-Kill curve data, *Staphylococcus aureus* was incubated with isolated fungal extract-1 (300 µl/ml) and fungal extract-2 (200 µl/ml) and *Klebsiella pneumonia* was treated with isolated fungal extract-1 (500 µl/ml) and fungal extract-2 (300 µl/ml) respectively for 24 h.

Freshly taken samples were fixed using a universal electron microscope fixative. Series dehydration steps were followed using ethyl alcohol and propylene oxide. The samples was then embedded in labeled beam capsules and polymerized.

Thin sections of cells exposed to extracts were cut using LKB 2209-180 ultra-microtome and stained with a saturated solution of urinyl acetate for 30 min and lead acetate for 2 min [22]. The procedure was applied to control cells not exposed to extracts and to extract-exposed cells. Electron Micrographs were taken using a Transmission Electron Microscope (JEM-100 CX Joel).

RESULTS AND DISCUSSION:

In a comparative study to differentiate the antibacterial effect between some bioactive extracts isolated from *Myrothecium species*, fermentation, characterization, screening experiments were done. Two out of ten fungal extracts tested showed a wide variation of antibacterial activity against the growth of *Klebsiella pneumoniae* with average zones of 27 and 19 mm respectively (Figure-1). However; two out of ten extracts inhibited the growth of MRSA under test with average inhibition zones of 35 and 25 mm respectively.

On the other hand, the other tested extracts did not show any antibacterial activity against MRSA under test (Figure-2). Ouattara [23] reported that eugenol was shown to have astronger bactericidal activity against *E. coli* and *K. pneumoniae* than some antibiotics. Nyfors [24] reported that polyphenols found in tea have been able to inhibit the growth of and/or kill the following pathogens: *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enteridis*, *Shigella flexnieri*, *Shigella dysenteriae*, *Streptococcus sobrinus*, *Lactobacillus rhammosus*, *Actinomyces viscosus*, *Listeria monocytogenes*, *Streptococcus salivarius*, *Streptococcus mitis* and *Vibrio chlorae*.

The most promising bioactive extract was MRP001 with MIC values 500 µl/ml. The present study showed that over all high potency in terms of MIC values was exhibited by the selected bioactive extracts (*Myrothecium* extracts) against the entire tested bacterial strains. MIC values of the extracts were in the following order: Extract-MRP001 (500µl/ml) > Extract-MRP014 (300 µl/ml) against the growth of *klebsiella pneumonia*, Whereas MIC values of the tested extracts against the growth of MRSA were Extract-MRP001 (300µl/ml) > Extract-MRP014 (200 µl/ml). MBC determination was based on the MIC, where the most promising plant extracts under test (MRP001 and MRP014) showed bactericidal effect against *Klebsiella pneumonia* with MBC values of 500 µl/ml and 300 µl/ml, and MBC values of 300 µl/ml and 200 µl/ml with gram-positive (MRSA) respectively (Figure-3). The time kill curve study showed that MRP001 and MRP014 exhibited a good and rapid bactericidal effect within 6-8 hrs for *Klebsiella pneumoniae* and within 2-4hrs for MRSA (Figure-4). Masatomo & Kazuko (2004) reported that a time kill study analysis showed that the survival of resting cells decreased immediately and rapidly with catechins, and the survival rate was < 1% after 4 hrs. However, a few colonies still survived after 24 hrs of culturing.

In the present investigation, a comparative study was done to evaluate the antibacterial activity of the most promising bioactive extracts (*Myrothecium spp.*) against *Klebsiella pneumoniae* and MRSA versus

commonly used antibiotics alone and in combined with the extracts. Seven antibiotic discs were selected, one was used for both gram-negative and gram-positive bacteria; ciprofloxacin (CIP). Two antibiotic discs for gram-negative bacteria were used: gentamycin (GM) and ciproflaxin(CIP). The discs used for gram-positive bacteria were cefoxitin (FOX), oxacillin (OX), ceftriaxone (CRO) and vancomycin (VA) (Abd-El Aal et al., 2007). With respect to *Klebsiella pneumoniae* there was an antagonist effect (Figure-5). On the other MRSA there was a highly synergistic increase due to the combination in all antibiotics, compared with inhibition zones of antibiotics alone except for CIP and CRO treated with bioactive extract against MRSA (Figure-6). The bioactive extracts were evaluated for qualitative determination of major constituents i.e. flavonoids, glycosides, phenols, saponins and steroids.

It was concluded that MRP001 and MRP014 extracts specifically and bioactive extracts in general may be potential sources of new and selective agents for the treatment of MRSA and *Klebsiella pneumoniae*. Further studies will be beneficial in providing data on the possible effects of these *Myrothecium* extracts if it is to be used as a relevant Antibiotic.

ACKNOWLEDGMENT

This work was supported by the Department of Biotechnology, SGB Amravati University, India Additional financial support at Shri. Shivaji College, Akola was provided by equipment grant. We thank Mr. Sudheer Adkar, Senior Director, a division of Microbial Biotechnology, IND, and Professor D. G. Bhadange, Department of Botany, for taxonomic identification for the fungus. We also thank Dr. S. G. Bhadange (Principal) for providing valuable support.

REFERENCES

[1] Rijnders M., Deurenberg R.H., Boumans M., Hoogkamp-Korstanje M., Beisser P., Stobberingh E.E. (2009). Antibiotic resistance of *Staphylococcus aureus* from ICUs in the Netherlands 1996 to 2006. Critical Care., 13: 305.

[2] Bocanegra-Garcia V., Camacho-Corona M., Ramirez-Cabrera M., Garza-Gonzatez G. (2009). The bioactivity of plant extracts against representative bacterial pathogens of the lower respiratory tract. BMC Res., 2: 95.

[3] Sajduda A., Dziadek J., Dela A., Zalewska Schonthaler N., Zwalska Z., Fadden J. (1998). DNA finger printing as an indicator of active transmission of multi drug resistant Tuberculosis in Poland. Int. Infect. Dis., 3: 12-17.

[4] Silbergeld E.K., Price L., Graham J. (2008). Antimicrobial Resistance and Human Health. Retrieved from Pew Commission on Industrial Farm Animal Production.

[5] Turner W.B., Aldridge D.C. (1983) Fungal Metabolites II; Academic Press: New York, pp 228-238.

[6] Cole R.J., Cox R.H. (1981) Handbook of Toxic Fungal Metabolites; Academic Press: New York, pp 152-263.

[7] Fenical W. (1993) Chemical studies of marine bacteria: developing a new resource. Chem Rev 93, 1673–1683.

[8] Faulkner D.J. (2001) Marine natural products. Nat Prod Rep18, 1–49.

[9] Rizwan M.R., Musaddiq M., Thakare P.V. (2012). In-vitro antimicrobial studies of isolated *Myrothecium* spp MRP001 against human pathogens. International Journal of Basic and Applied Medical Sciences. 2(3): 228-236.

[10] Buchanan R., Gibbson N. (1974). Bergey's Mannual of determinative Bacteriology. 8th ed., Williams and willlkins, Baltimore.

[11] Madigan, M.; Martinko, J. and Brock, T. 2006. Brock Biology of microorganisms. Pearson Prentice Hall., p: 767-771.

[12] Mahon, C.; Smith, L. and Burns, C. 1998. An introduction to Clinical Laboratory Science. W.B. Saunders Company., 37-43.

[13] Ifeoma E., De N. (2002). Antibacterial effect of components of the bark extract of neem (*Azadirachta indica* A. Juss). Technological Development Journal 8 23-28.

[14] Rizwan M.R., Musaddiq M., Thakare P.V. (2012). In-vitro antimicrobial studies of isolated *Myrothecium* spp MRP001 against human pathogens. International Journal of Basic and Applied Medical Sciences. 2(3): 228-236.

[15] Falahati M., Tabrizib N.O., Jhanyani F. (2005). Anti Dermatophytes activities of Eucalyptus camaldulensis in comparison with griseofulvin; IJPT., 4:80-83.

[16] NCCLS. (2000). National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grows aerobically, 5th edn. Approved StandardM7-A5.Wayne, PA.

[17] Ellof J. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta.Medica., 64: 711-713.

[18] Rota M., Herrera A., Martinez R., Sotomayor J., Jordan M. (2008). Antimicrobial activity and chemical composition of thymus vulgaris, Thymus zygis and thymus hyemalis essential oils. Food control., 19: 681-687.

[19] Yin M., Chang H., Tsao S. (2002). Inhibitory effect of aqueous garlic extract, garlic oil and four diallylsulphides against four enteric pathogens. J. Food Drug Anal., 10: 120-126.

[20] Abd-El Aal A.M., El-Hadidy M.R., El-Mashad N.B., El-Sebaie A.H. (2007). Antimicrobial effect of bee honey in comparison to antibiotics on organisms isolated from infected burns. Applied Microbiol., 93: 857-863.

[21] Sibi G., Apsara V.K., Dhananjaya K.R., Ravikumar H., Mallesha. (2013). Phytochemical and Antibacterial Properties of Spices against Food Borne Bacteria with Special Reference to Parmelia Per. G.J.B.B., 2 2: 145-149.

[22] McDowell E.M, Trump B.F. (1967). Histoligic fuxative suitable for diagnostic light and electron microscopy. Arch pathol lab., 10: 405-413.
[23] Ouattara B., Simard R.E., Holley R.A., Piette G.J.P., Begin, A. (1997). Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int J Food Microbiol., 37, 155-162.
[24] Nyfors S., Syrjanen R., Kononen E. (2004). Impact of antimicrobial exposure and beta lactamase producing bacteria on salivary beta lactamase activity in infancy. Intenational Journal of Antimicrobial Agents., 24 5: 463-467.

Figure-1: Agar Well diffusion test of *Klebsiella pneumoniae* against; (a) Control, (b) MRP001 and (c) MRP014

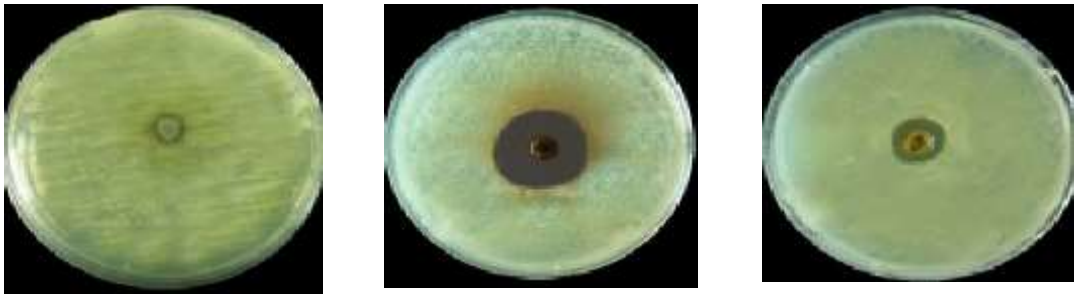

Figure-2: Agar Well diffusion test of MRSA against; (a) Control, (b) MRP001 and (c) MRP014

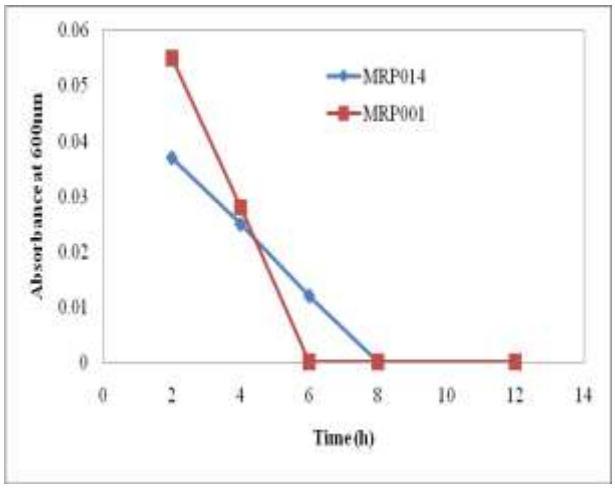

Figure-3: Time kill curve of *Klebsiella pneumoniae* treated with MRP001 and MRP014 Extracts.

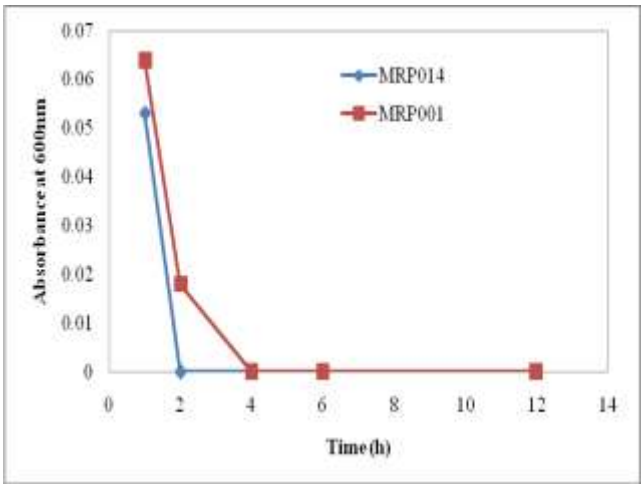

Figure-4: Time kill curve of MRSA treated with MRP001 and MRP014 Extracts.

IJBST

ISSN: 0974 – 3987
IJBST (2014), 7(4):25-30

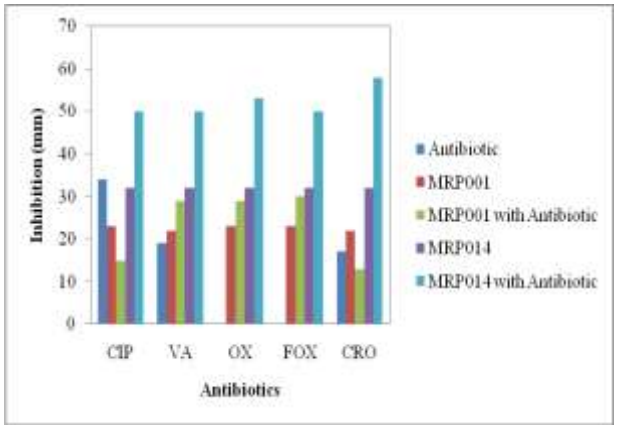

Figure-5: The effect of MRP001, MRP014 and their combined action with antibiotics against MRSA

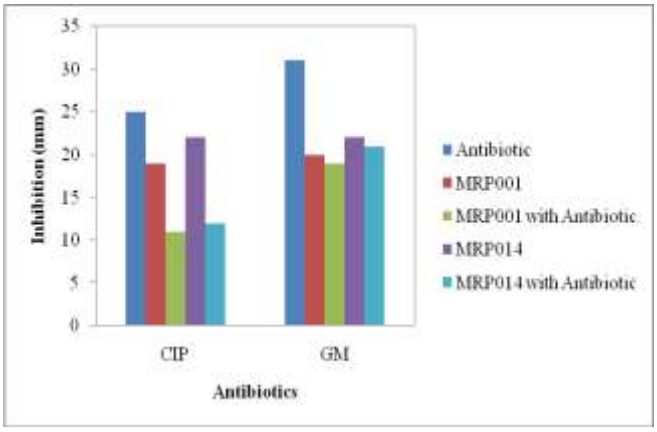

Figure-6: The effect of MRP001, MRP014 and their combined action with Antibiotics against *Klebsiella pneumoniae*